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PHYLOGENETIC RELATIONSHIPS AMONG GIANT PANDA AND RELATED SPECIES BASED ON RESTRICTION SITE VARIATIONS IN rDNA SPACERS*

LAN Hong[①②] WANG Wen[①]

(①*Lab. of Cellular and Molecular Evolution, Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming* 650223)
(②*Current address: Department of Human Oncology, University of Wisconsin-Madison, K4/347 CSC, 600 Highlans Avenue, Madison, WI* 53792. *U. S. A.*)

Abstract In this study, non-radioactive Digoxigenin labeled ribosomal DNA (rDNA) probes were used for Southern blotting analysis to study the molecular phylogeny of the giant panda and related species. Restriction maps in the regions of rDNA spacers were compared between giant panda (*Ailuropoda melanoleuca*), lesser panda (*Ailurus fulgens*), Asiatic black bear (*Selenarctos thibetanus*), sun bear (*Helarctos malayanus*), raccoon (*Procyon lotor*) and lynx (*Felis Lynx*). Phylogenetic trees for these species were constructed using maximum likelihood and parsimony method. The results show that in respect to rDNA RFLPs, the giant panda is more closely related to bear than to lesser panda; while the lesser panda is slightly related to the raccoon.

Key words Arctoidea, The giant panda, Ribosomal DNA restriction maps, Phylogenetic relationships

1 Introduction

The giant panda (*Ailuropoda melanoleuca*) is an endemic species of China and might be the most popular wild animal in the world. As matched with its popularity, numerous studies have been done on the morphology, ecology and behavior of the giant panda. However, although the giant panda is regarded as a "living fossil" in zoology and paleontology, its evolutionary relationships with other related species in Arctoidea, such as with the bear or the lesser panda, have been a biological puzzle (Van Valen, 1986). Most classification studies vary in placing the giant panda as a bear, a relative of the lesser panda, or a distinct family (Van Valen, 1986; Zhang *et al.*, 1993).

In the past decades, traits related to anatomy, behavior, karyology, immunological distances, isozyme electrophoresis and DNA hybridization, were used to clarify the classification and evolution of the giant panda (Sarich, 1973; O'Brien *et al.*, 1985; Van Valen, 1986). In 1985, O'Brien *et al.* analyzed the molecular genetic data obtained from four different ge-

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netic methods and seemed to have solved the dilemma by concluding that the giant panda is closer to the bear. Subsequently, research with hemoglobin sequences (Tangle *et al.*, 1986) and mitochondrial DNA (mtDNA) RFLPs (Zhang *et al.*, 1991), placed the giant panda as a close relative of the lesser panda. More recent analyses on mtDNA sequences (Zhang *et al.*, 1993) as well as maximum likelihood analysis of α-hemoglobin and β-hemoglobin sequences (Hashimoto *et al.*, 1993) have again showed that the giant panda is closer to bear. More data, especially from nuclear DNA, would aid in consolidating the "giant panda-bear" relationship. To our knowledge, the nuclear DNA RFLPs of the giant panda and related species have not been studied yet.

Ribosomal RNA (rRNA) genes are multigene families, and generally there are several hundred copies in one mammalian genome (Arnheim *et al.*, 1983). The copies are usually distributed in tandem repeats at several different sites on chromosomes. Each rDNA repeating unit is composed of three rRNA genes, 28*S*, 5.8*S* and 18*S* RNA, which are separated from each other by spacers. The spacer regions, especially the non-transcriptional spacer between 28*S* and 18*S* RNA genes, are known to evolve rapidly, and considerable RFLPs are apparent between populations and species (Arnheim *et al.*, 1983; Wilson *et al.*, 1984; Hillis *et al.*, 1986, 1988; Suzuki *et al.*, 1990, 1994). Because of the strong forces that encourage homogenization within a genome as well as within a population, sequence homogeneity is maintained within a population (Dover, 1982). Therefore, small samples could usually be a good representative in phylogenetic analysis of a species or a population (Hillis *et al.*, 1986). These features described above make the analysis of rDNA RFLPs a good approach for phylogenetic studies at the species, genus and even family level, and a lot of achievements have been reached (Arnheim *et al.*, 1980; Wilson *et al.*, 1984; Hillis *et al.*, 1986, 1988; Suzuki *et al.*, 1990, 1994; Allard *et al.*, 1991; Wall *et al.*, 1992; Lan *et al.*, 1993).

In this study we compared the restriction maps for 13 restriction enzymes on the rDNA spacer regions of the giant panda and related species using an non-radioactive DNA labeling and detecting method (Boehringer Mannheim). Phylogenetic reconstruction using maximum likelihood and maximum parsimony were conducted with the resultant matrix of sites. The results suggest that phylogenetic relationships among giant panda, lesser panda, bear and raccoon are similar to those based on mtDNA and hemoglobin sequence comparisons (Zhang *et al.*, 1993; Hashimoto *et al.*, 1993).

2 Materials and Methods

2.1 Samples

Blood samples of the giant panda *Ailuropoda melanoleuca* and the raccoon *Procyon lotor* were provided by the Chengdu Zoo, China. Samples of the other species, lesser panda *Ailurus fulgens*, Asiatic black bear *Selenarctos thibetanus*, sun bear *Helarctos malayanus* and lynx *Felis lynx*, were collections of the Kunming Cell Bank, the Chinese Academy of Sciences. We used two individuals of giant panda and lesser panda in this study, and one for the remaining species.

2.2 Southern hybridization analysis

DNA was isolated from bloods or cultured fibroblasts according to the method described by Sambrook *et al.* (1989). Genomic DNA (2 − 5 mg) was digested with 5 U restriction en-

donuclease at 37℃ overnight. Completely digested DNA was separated in a 15 symbol 180 \ f "Symbol" \ s 12× | 20 cm 0.7% agarose gel at 1 V/cm overnight in TAE buffer, denatured, neutralized, and subsequently transferred to a nylon membrane (Boehringer Mannheim, # 14172420) by Southern blotting in 10 symbol 180 \ f "Symbol" \ s 12× | standard saline citrate (SSC).

The clones of the three rDNA probes, namely pHr21Aa for 18S, pHrEP for 28S and pHr14E3 for INT were originally developed by Dr. M. Muramatsu and were obtained from Japanese Cancer Research Bank (JCRB). The positions of each cloned probe in the rDNA array are indicated in Fig. 1a. For probe labeling and signal detection, we used the Dig DNA Labeling and Detection Kit (Boehringer Mannheim, #1093657), and all the protocols followed the manufacture's specifications.

2.3 Construction of restriction maps

Restriction maps of the rDNA spacers for each species were constructed based on hybridization patterns with three rDNA probes after appropriate single and double digestion. We compared our results with the conserved restriction maps of the rDNA coding region constructed in previous analyses (Hillis *et al.*, 1986). Because that the two *Eco*R I sites on the 3'ends of both 28S and 18S rDNA coding regions (Fig. 1a) are conserved in vertebrates (Suzuki *et al.*, 1990), cleavage site of *Eco*R I on the 28S downward spacer was determined according to the patterns of *Eco*R I single digestion after hybridization with 28S probe. Sites of other enzymes inside the region were determined subsequently by double digestion of *Eco*R I with each of the given enzymes. For those sites outside this region, double digestions will yield the same pattern of a single digestion with *Eco*R I, and hence were determined by single digestion and by referring their conserved cleavage sites in coding region. The same procedure was used to map those sites upward the 18S gene. INT probe was used to cleavage sites in the internal spacer region.

2.4 Phylogenetic analysis

The presence and absence of a site on a given position of the restriction map were represented by "1" and "0" characters, respectively. The restriction sites among the maps were aligned by introducing length variations into the region next to the 3'end of 28S gene (Suzuki *et al.*, 1994) to maximize sequence similarity. Thus we obtained a matrix of characters for phylogenetic analysis (Table 1). The data set serve to construct a maximum likelihood tree using RESTML program in PHYLIP 3.51c package (Felsenstein, 1993) as well as parsimony trees using PAUP 3.1.1 software (Swofford, 1991). In all the phylogenetic analyses, the lynx was used as an outgroup.

Table 1 The "0, 1" characters of restriction sites of rDNA spacers in the giant panda and related species

Animal	Characters		
	28S	18S	INT
Black bear	0000111110100100001000000001000010000110010000000	10011010100000000001111000101001	1
Sun bear	0000111110100100001000000001000000000110010010000	10011010100000000001111000101001	1
Giant panda	0001010110000100000010000000000001110010000110011	01001010100001001000101000100111	0
Lesser panda	1111000001111011100000010000100000001001000000000	00000001011011100110000100011001	0
Raccoon	0001000010000000011001010011000011010000000000000	00000110001101101000000001110011	0
Lynx	0001000010000001000101101000010010010000011010100	00100000000111111000000011111011	0

Note: "1" indicates the presence of a site, and "0" the absence of a site.

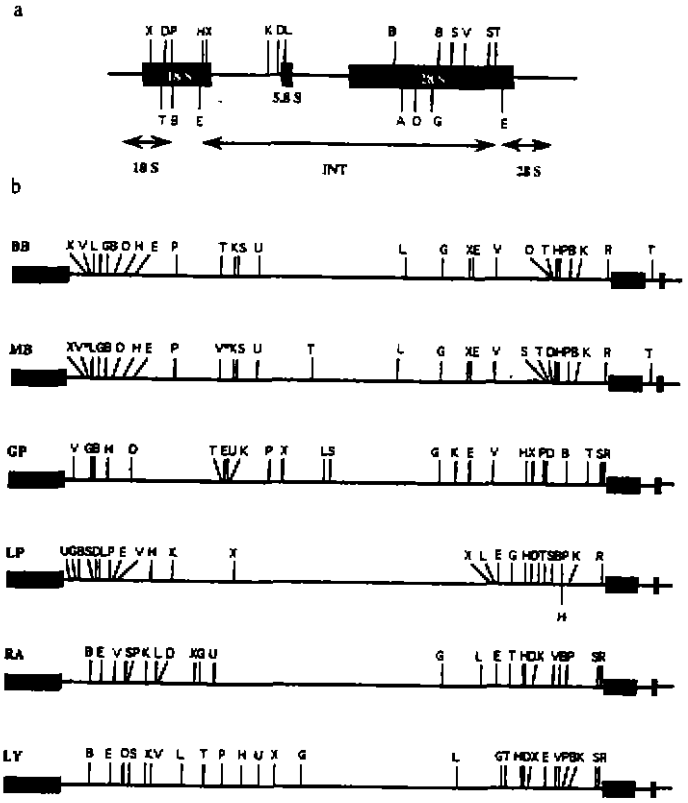

Fig.1 Restriction maps of the rDNA coding region (a) and spacer regions (b) of the giant panda and related species

The black boxes are the genes that encode 18S, 28S and 5.8S rRNA, respectively. Positions of probes are also shown by arrows. With respect to the restriction sites in the spacers, only those nearest the distal end of the gene for 18S and 28S rRNA are shown. Asterisks indicate polymorphic sites within the genome of a given animal, while the *Hin*dⅢ site downward the horizontal line in the map of the lesser panda is that shows intraspecific variation. Abbreviation of species: BB, Asiatic black bear; MB, sun bear; GP, giant panda; LP, lesser panda; RA, raccoon; LY, lynx. Abbreviations of restriction sites: A: *Stu* Ⅰ, B: *Bam*HⅠ, D: *Dra* Ⅰ, E: *Eco*RⅠ, G: *Bgl* Ⅱ, H: *Hin*dⅢ, K: *Kpn* Ⅰ, L: *Bcl* Ⅰ, P: *Pst* Ⅰ, S: *Sac* Ⅰ, T: *Bst*EⅡ, V: *Pvu* Ⅱ, X: *Xba* Ⅰ.

3 Results and Discussion

The rDNA restriction maps of the giant panda and related species are showed in Fig.1. Eighteen restriction enzymes, *Apa* Ⅰ, *Ava* Ⅰ, *Bam*HⅠ, *Bcl* Ⅰ, *Bgl* Ⅱ, *Bst*EⅡ, *Cla* Ⅰ, *Dra* Ⅰ, *Eco*RⅠ, *Eco*RⅤ, *Hin*dⅢ, *Hpa* Ⅰ, *Kpn* Ⅰ, *Pst* Ⅰ, *Pvu* Ⅱ, *Sac* Ⅰ, *Stu* Ⅰ, *Xba* Ⅰ, were used in this study. No maps for *Apa* Ⅰ, *Ava* Ⅰ, *Cla* Ⅰ, *Eco*RⅤ and *Hpa* Ⅰ were obtained due to either one or no cleavage site on the rDNA repeat unit. Therefore the restriction maps contain sites for 13 enzymes. By using the INT probe, we detected an additional *Bst*EⅡ site in the internal spacer regions of the Asiatic black bear and the sun bear. As regard to the intraspecific polymorphism, only one *Hin*dⅢ site was found differ between the two individuals of the lesser pandas in the spacer region near the 18S rRNA gene (Fig.1). The diverged lesser panda was not used in phylogenetic analysis. No site was found differ between the two giant pandas, hence only one individual is show in Fig.1. The *Pvu* Ⅱ pattern of the sun bear always

shows two bands when hybridized with 28S probe even after excessive digestion, therefore we regard it as a intra-genome heterozygote rather than partial digestion. Both the two sites are showed in the map but the variant one was not taken into account for phylogenetic analysis.

Recent studies showed that the evolutionary rates of both hemoglobin and mitochondrial genes among different lineages in the Arctoidea are different (Hashimoto *et al.*, 1993; Zhang *et al.*, 1993), therefore when analysing the restriction site data, it is important to employ a method which does not necessarily assumes the constancy of evolutionary rate among branches. In this study, we used the restriction site maximum likelihood method (RESTML) in PHYLIP version 3.51c to construct the phylogenetic tree for the 6 species from the information relating to the presence or absence of each restriction site (Table 1). A single tree came out with Ln Likelihood = −173.17 (Fig.2A). In this phylogenetic tree, the Asiatic black bear and the sun bear are closely related, then is the giant panda, the lesser panda and the raccoon form an other branch.

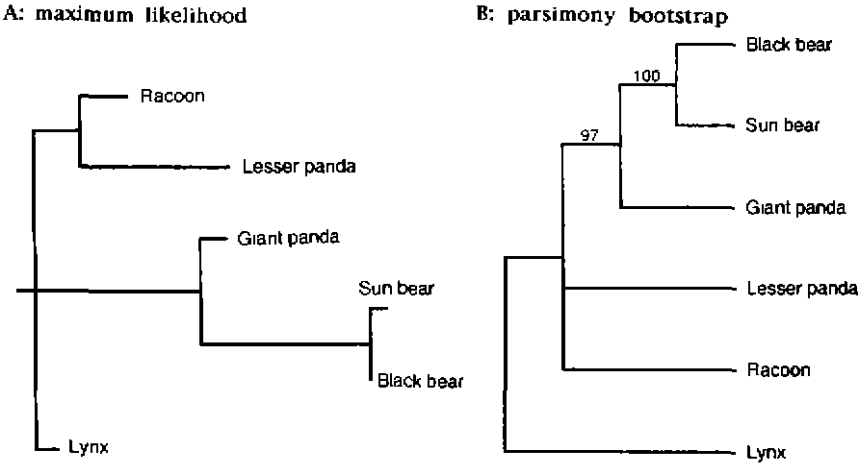

Fig.2 Maximum likelihood tree (A) and parsimony tree (B) for 5 species of Arctoidea
The lynx is used as an outgroup. The horizontal length of each branch in A is proportional to the estimated divergence. The figures above some branches in B are the percentage of trees retaining the branch based on 1 000 bootstrap replicates.

In addition, we also used exhaustive search option to construct parsimony trees by use of PAUP 3.1.1. Three shortest trees were found which have a branch length of 96. These three parsimony trees differ in the relation between the lesser panda and the raccoon. In one tree, the lesser panda is at the basal branch while in another the raccoon at the basal branch. The third parsimony tree which has the same topology with the likelihood tree (Fig.2A) clusters both of them in the basal branch. Fig.2B show the consensus tree for the three parsimony tree with the support percentage which retains a certain branch in 1 000 bootstrap replicates. This consensus tree again strongly supports that the giant panda is closer to bear than lesser panda, which is consistent with most of the previous research. (O'Brien *et al.*, 1985; Zhang *et al.*, 1993; Hashimoto *et al.*, 1993).

The phylogenetic placement of the lesser panda is another interesting question in Arctoidea systematics. O'Brien *et al.* (1985) suggested that it might be slightly linked to the raccoon.

The likelihood tree and one of the three parsimony trees obtained in the present study shows that the lesser panda is closer to the raccoon than to the bear-giant panda branch (Fig.2). Although other two parsimony trees failed to cluster the lesser panda and the raccoon into one clade, their branches are close one another indeed in the trees (not shown). Therefore, we are cautiously prone to uhe view that the lesser panda is slightly related to the raccoon.

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大熊猫及其近缘种 rDNA 序列变异和系统进化关系

兰　宏①②　王　文①

（①中国科学院昆明动物研究所细胞与分子进化开放研究实验室　昆明　650223）
（②威斯康星大学人类肿瘤学系　迈德林 WI 53972 USA）

摘　要　应用 rDNA 间隔区 Southern 转移技术研究大熊猫及其近缘种的分子系统关系。通过比较大熊猫、小熊猫、黑熊、马来熊、浣熊和猪獾的 rDNA 间隔区限制性内切酶图谱，用最大似然法和简约法构建它们的分子系统树。结果表明大熊猫与熊具有较近的亲缘关系，与小熊猫和浣熊的亲缘关系较远。

关键词　熊超科，大熊猫，rDNA 限制性图谱，系统发育

中图分类号　Q959.838

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* 会议消息 *
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“第十八届国际遗传学大会昆明卫星会议”简介

“第十八届国际遗传学大会昆明卫星会议”于 1998 年 8 月 16 日～18 日在云南省昆明市举行。本次会议由中国科学院昆明动物研究所与云南省遗传学会联合主办，会议主席由我国著名植物学家、中国科学院院士吴征镒先生担任，秘书长为中国科学院昆明动物研究所副所长张亚平研究员。

本次会议是一次高水平的国际性学术会议，有来自中国、美国、日本、英国、以色列、澳大利亚、加拿大以及中国香港特别行政区、台湾省等 10 余个国家和地区的知名专家和学者约 200 人参加。其中国外代表 40 余人，国内代表约 170 人。会议共收到论文摘要 176 篇，内容涉及动物、植物、微生物和人类遗传多样性等诸多学科。

8 月 16 日晚，会议主席吴征镒院士在昆明连云宾馆主持了隆重的迎宾晚宴，中国科学院院士吴旻、吴常信、陈竺等，国外著名学者 Oliver A. Ryder、Ranajit Charkraborty、Rober K. Moizis、Yun-xin Fu、吴仲仪和李文雄等应邀出席了宴会。会议于 17 日在云南大学开幕，云南省副省长李汉柏出席了开幕式。新华社、《中国科学报》、云南电视台等新闻单位的记者也参加了开幕式。开幕式结束后，围绕会议主题“遗传学和生物多样性保护”，中外学者作了 9 个大会报告。著名科学杂志《Nature》的编辑 David Dickson 先生也应邀作了报告。18 日，与会专家学者分为人类、动物、植物和微生物 3 个专题组，就种群遗传、进化遗传、保护遗传等内容进行了广泛而深入地探讨。共有 62 人作了专题报告，约 130 人次参加了墙报交流。大会于 18 日晚圆满结束。此外，围绕生物多样性资源保护，于 19～22 日，会议还组织了会后考察，为中外科学家充分了解云南丰富的生物资源、进一步认识云南在开展分子进化和遗传多样性研究中的独特地位提供了更多的交流机会。

会议期间和会后，数十名中外学者来到昆明动物研究所参观和交流。美国、日本等国家的几个研究小组与该所科学家进行了深入交谈，商讨今后合作的方向与有关事宜，为昆明动物研究所今后加强国际间的交流与合作拓宽了渠道。

本次会议的举行，对于促进世界范围内的生物遗传多样性保护与合作、推动人类与生物进化和遗传学理论的发展、以及为我国保护生物学与进化生物学研究在世界范围内赢得一席之地，都将起到积极的作用。

罗　静

（中国科学院昆明动物研究所细胞与分子进化开放研究实验室　650223）